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Original Article

Validation of reference genes for the normalization of qRT-PCR expression studies in head and neck squamous cell carcinoma cell lines treated by different chemotherapy drugs

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Abstract: Selection of stably expressed reference genes is crucial for data evaluation of quantitative real-time polymerase chain reaction (qRT-PCR) assay via relative quantification method. In this present study, we were committed to selecting the optimal reference genes for the investigation of target gene expression profiling in head and neck squamous cell carcinoma (HNSCC). Two cell lines, CNE and FaDu, were investigated. Both of them were treated by 5 different common chemotherapy drugs (CTX, DDP, PTX, VCR, and 5-FU), respectively. 12 reference genes (*GAPDH*, *ACTB*, *B2M*, *18S rRNA, ALAS1*, *RPL29*, *TBP*, *HPRT1*, *PUM1*, *HMBS*, *PPIA,* and *GUSB*) which are commonly used in the qRT-PCR method for analysis of gene expression were investigated via geNorm, NormFinder, and BestKeeper programs. From the results, we conclude that *ALAS1, ALAS1,* and *GAPDH* are the optimal reference genes for study of the total group (both CNE and FaDu cell lines), CNE cell line, and FaDu cell line, respectively. If using multiple reference genes to improve accuracy, for the total group, the recommended combination was *ALAS1* + *HMBS* + *HPRT1*. For the CNE cell line, the recommended combination was *ALAS1* + *RPL29*. For the FaDu cell line, the recommended combination was *GAPDH* + *ALAS1* + *HPRT1*. Our findings provide a suitable approach via qRT-PCR that can be applied to investigate the pharmacological effects and the molecular mechanism of chemotherapy drugs on HNSCC.

Keywords: Quantitative real-time polymerase chain reaction, relative quantitation, reference gene, head and neck squamous cell carcinoma, chemotherapy treatment

Introduction

Relative quantification analysis is a common, accurate, and easy to operate method which is widely used in many gene expression investigations of molecular biological research. A stably expressed internal control gene is used as a standard to measure and compare the relative expression levels of target genes in the same biological sample. Therefore, in relative quantification analysis, it is very important to evaluate and identify appropriate reference genes. Only the reference gene which is expressed stably under various experimental conditions can be considered as an ideal reference gene [1-3]. However, there are more and more studies that have demonstrated that the expression levels of most reference genes, commonly used in many studies, such as β-actin (ACTB), ribosomal RNA (18S rRNA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are variable across cell types, or in distinct tissues [4-7], even between treatments of the same tissue [8-10]. Thus, it is very important to identify and choose optimal reference genes before studying different kinds of cell types and tissues on the gene expression level through relative quantification analysis.

Each year, more than 500,000 head and neck squamous cell carcinoma (HNSCC) cases are diagnosed all over the world [11]. Unfortunate-

Table 1. Summary of reference genes used in the present study

Symbol	Official full name	Accession Number	Primer sequence[a]	Product size (bp)	Efficiency
18S	18S ribosomal RNA	NM_10098.1	F: CGGCTACCACATCCAAGGAA	186	2.45
			R: GCTGGAATTACCGCGGCT		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.5	F: GACAGTCAGCCGCATCTTCT	127	2.14
			R: TTAAAAGCAGCCCTGGTGAC		
B2M	Beta-2-microglobulin	NM_004048.2	F: AGCGTACTCCAAAGATTCAGGTT	306	2.19
			R: ATGATGCTGCTTACATGTCTCGAT		
ACTB	Actin, beta	NM_001101.3	F: AGAAAATCTGGCACCACACC	173	2.43
			R: TAGCACAGCCTGGATAGCAA		
ALAS1	5'-aminolevulinate synthase 1	NM_000688.5	F: GGCAGCACAGATGAATCAGA	150	2.08
			R: CCTCCATCGGTTTTCACACT		
GUSB	Glucuronidase, beta	NM_000181.3	F: AGCCAGTTCCTCATCAATGG	160	2.31
			R: GGTAGTGGCTGGTACGGAAA		
HPRT1	Hypoxanthine phosphoribosyl transferase 1	NM_000194.2	F: GACCAGTCAACAGGGGACAT	132	2.46
			R: CCTGACCAAGGAAAGCAAAG		
HMBS	Hydroxymethylbilane synthase	NM_000190.3	F: AGTGTGGTGGGAACCAGC	144	2.39
			R: CAGGATGATGGCACTGAACTC		
PPIA	Peptidylprolyl isomerase A	NM_021130.4	F: AGACAAGGTCCCAAAGAC	118	2.37
			R: ACCACCCTGACACATAAA		
PUM1	Pumilio RNA-binding family member 1	NM_001020658.1	F: CAGGCTGCCTACCAACTCAT	211	2.41
			R: GTTCCCGAACCATCTCATTC		
RPL29	Ribosomal protein L29	NM_000992.2	F: GGCGTTGTTGACCCTATTTC	120	2.42
			R: GTGTGTGGTGTGGTTCTTGG		
TBP	TATA box binding protein	NM_003194.4	F: TGCACAGGAGCCAAGAGTGAA	132	2.33
			R: CACATCACAGCTCCCCACCA		

[a]F: forward primer; R: reverse primer.

ly, approximately two-thirds of these patients are in advanced stages [12]. Radiotherapy or chemotherapy are the conventional treatments for the HNSCC patients. However, solving the chemotherapy multidrug resistance problem has become the focus of the study [13]. Among the chemotherapy strategies of HNSCC, Cyclophosphamide (CTX), cis-Dichlorodiamineplatinum (II) (DDP), Paclitaxel (PTX), Vincristine (VCR), and 5-Fluorouracil (5-FU) are the most widely used chemotherapy drugs [14-17].

GAPDH, *ACTB*, *B2M*, *18S rRNA, ALAS1*, *RPL29*, *TBP*, *HPRT1*, *PUM1*, *HMBS*, *PPIA,* and *GUSB* are commonly used references which have been validated and suggested as suitable reference genes in the studies of certain other cancers [18, 19]. In order to determine the optimal reference genes in gene expression studies of HNSCC by relative quantification analysis, these 12 candidate genes were validated in the present study, both in CNE (human nasopharyngeal carcinoma cell line) and FaDu (pharyngeal squamous cell carcinoma cell line).

Materials and methods

Cell culture and treatment

CNE and FaDu cell lines were donated by the Research Center of Second Hospital, Jilin University. They were cultivated according to the recommendation of the supplier, in IMDM containing 10% FBS with 100 units of Penicillin-Streptomycin for CNE, and RPMI 1640 containing 10% FBS with 100 units of Penicillin-Streptomycin for FaDu, maintained at 37°C in 5% CO_2 humidified atmosphere.

CNE and FaDu cell lines were treated at a confluency of 80-90% with CTX (final concentration was 2.00 mg/mL for CNE and FaDu), DDP (final concentration was 0.45 μg/mL for CNE and 50 μg/mL for FaDu), PTX (final concentration was 0.84 μg/mL for CNE and 50 μg/mL for FaDu), VCR (final concentration was 2.00 μg/mL for CNE and 6.18 μg/mL for FaDu), and 5-FU (final concentration was 100 μg/mL for CNE and 50 μg/mL for FaDu), respectively. Cells treated with medium only were used as

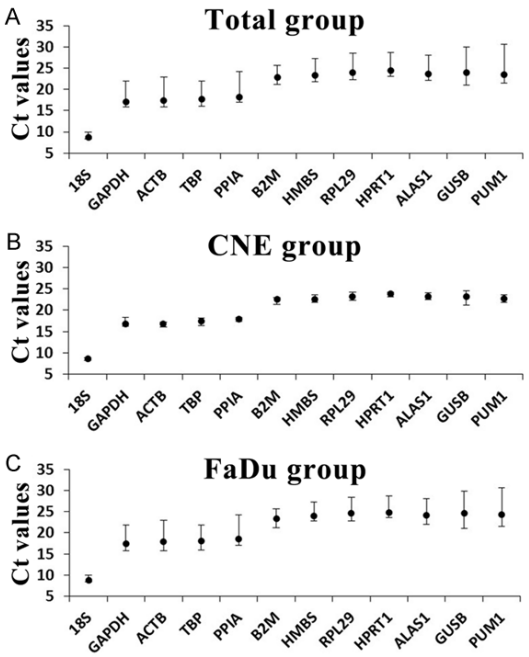

Figure 1. Cp values of the candidate reference genes in the total group (A), the CNE group (B), and the FaDu group (C). Dots represent the average Cp values of each candidate reference gene; bars represent the minimal to maximal Cp values.

the control. All cells were collected 24 hours after the treatment.

RNA extraction and reverse transcription

TRIzol Reagent (Invitrogen Life Technologies, USA) was used for extracting total RNA from each group. NanoDrop 2000 (Thermo Fisher Scientific, USA) was used for measuring the concentration and purity of the isolated RNA. DNase I was used for eliminating the residue genomic DNA. 1 μg of total RNA was used for reverse transcription reaction by M-MuLV First Strand cDNA Synthesis kit (Sangon, China). All operations were carried out according to the instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The primer sequences of 12 candidate reference genes are listed in **Table 1**. All of these primer sequences were based on previous studies [20]. qRT-PCR was performed on Roche LightCycler 480 instrument (Roche, Germany). 2×SG Fast qPCR Master Mix (Sangon, China) was used for qRT-PCR, as previously described [20, 21]. The Cp value data were pre-converted into relative quantities (Q) using the equation $Q = 2^{-\Delta Cp}$ [22] for the following statistical analysis.

Amplification efficiency

A random pool of cDNA from the samples was employed for determining the amplification efficiency of qRT-PCR. The cDNA was diluted into 4 series, ranging from the original concentration to 1/1000 of the concentration. The equation $E = 10^{-1/slope}$ was used for calculating the amplification efficiency.

Statistical analysis

The samples were divided into 3 groups: total group (taking both CNE and FaDu cell lines into consideration), CNE group, and FaDu group. Three software programs, geNorm [23], NormFinder [24], and BestKeeper [25] were utilized to assess the stability of the reference genes as previously described [3, 6, 7, 21].

Results

The Cp values of candidate reference genes

The expression profiles of the candidate reference genes were reflected by Cp values. A higher Cp value indicates a lower amount of expression level. In **Figure 1**, we can see that the Cp values of all the samples range between 8.25 (*18S rRNA*) and 30.70 (PUM1).

The expression stability of candidate reference genes

GeNorm: From the results of geNorm in the total group, *HMBS* and *HPRT1* had the lowest M-values which means that they are the most stably expressed candidate genes in both CNE and FaDu cells treated with 5 types of chemotherapy drugs. In the CNE group, *ALAS1* and *RPL29* had the lowest M-values; in FaDu group, *GAPDH* and *ALAS1* had the lowest M-values (**Table 2**). A combination of 4 reference genes, with V4/5 of 0.132 and 0.149, was optimal for the total group and FaDu group to further improve the normalization; 2 reference genes combined together was optimal for the CNE group, with V2/3 of 0.128 (**Figure 2**).

NormFinder: From the results of NormFinder, we can see that the combination of *ALAS1* and *HMBS* has the lowest Stability value (0.079) which means that the combination of *ALAS1* and *HMBS* is the optimal choice for the total

Table 2. Twelve candidate reference genes ranked by different software

Rank (weight)	Program							
	geNorm		NormFinder		BestKeeper		Final ranking	
	Gene	M-value	Gene	Stability value	Gene	R	Gene	Geo Mean
Total group								
1	*HMBS*	0.595	*ALAS1*	0.110	*ACTB*	0.969	*ALAS1*	1.817
2	*HPRT1*	0.595	*HMBS*	0.113	*GAPDH*	0.964	*HMBS*	2.000
3	*ALAS1*	0.670	*GAPDH*	0.127	*ALAS1*	0.964	*HPRT1*	2.714
4	*GAPDH*	0.702	*HPRT1*	0.140	*HMBS*	0.956	*ACTB*	3.107
5	*PPIA*	0.735	*ACTB*	0.169	*HPRT1*	0.938	*GAPDH*	3.302
6	*ACTB*	0.767	*B2M*	0.198	*B2M*	0.932	*PPIA*	6.542
7	*TBP*	0.812	*PPIA*	0.201	*PPIA*	0.917	*B2M*	6.604
8	*B2M*	0.850	*TBP*	0.208	*GUSB*	0.917	*TBP*	7.958
9	*RPL29*	0.889	*RPL29*	0.222	*TBP*	0.91	*GUSB*	9.166
10	*GUSB*	0.962	*18S rRNA*	0.281	*RPL29*	0.858	*RPL29*	9.322
11	*PUM1*	1.016	*GUSB*	0.304	*18S rRNA*	-	*18S rRNA*	10.954
12	*18S rRNA*	1.081	*PUM1*	0.317	*PUM1*	-	*PUM1*	11.489
CNE group								
1	*ALAS1*	0.222	*18S rRNA*	0.199	*HPRT1*	0.870	*ALAS1*	2.410
2	*RPL29*	0.222	*ALAS1*	0.224	*GAPDH*	0.748	*RPL29*	2.621
3	*B2M*	0.333	*B2M*	0.224	*RPL29*	0.572	*18S rRNA*	3.420
4	*18S rRNA*	0.384	*ACTB*	0.282	*HMBS*	0.549	*HPRT1*	3.659
5	*ACTB*	0.474	*HMBS*	0.292	*ACTB*	0.526	*B2M*	4.160
6	*HMBS*	0.524	*RPL29*	0.313	*PPIA*	0.522	*ACTB*	4.642
7	*HPRT1*	0.572	*HPRT1*	0.344	*ALAS1*	0.450	*HMBS*	4.932
8	*PPIA*	0.621	*GAPDH*	0.456	*B2M*	0.420	*GAPDH*	5.241
9	*GAPDH*	0.660	*PPIA*	0.497	*TBP*	0.308	*PPIA*	7.560
10	*TBP*	0.697	*TBP*	0.518	*18S rRNA*	0.250	*TBP*	9.655
11	*PUM1*	0.743	*PUM1*	0.599	*PUM1*	-	*PUM1*	11.000
12	*GUSB*	0.787	*GUSB*	0.607	*GUSB*	-	*GUSB*	12.000
FaDu group								
1	*GAPDH*	0.510	*GAPDH*	0.164	*GAPDH*	0.992	*GAPDH*	1.000
2	*ALAS1*	0.510	*HMBS*	0.260	*HPRT1*	0.983	*ALAS1*	2.289
3	*HPRT1*	0.616	*ALAS1*	0.313	*ACTB*	0.978	*HPRT1*	2.884
4	*HMBS*	0.684	*HPRT1*	0.340	*ALAS1*	0.976	*HMBS*	3.420
5	*PPIA*	0.749	*PPIA*	0.486	*HMBS*	0.974	*ACTB*	5.278
6	*TBP*	0.798	*TBP*	0.502	*B2M*	0.956	*PPIA*	5.593
7	*ACTB*	0.850	*ACTB*	0.549	*PPIA*	0.955	*TBP*	6.604
8	*RPL29*	0.926	*B2M*	0.747	*TBP*	0.947	*B2M*	7.560
9	*B2M*	1.005	*RPL29*	0.776	*GUSB*	0.925	*RPL29*	8.963
10	*GUSB*	1.102	*GUSB*	0.882	*RPL29*	0.868	*GUSB*	9.655
11	*PUM1*	1.165	*PUM1*	0.953	*PUM1*	-	*PUM1*	11.000
12	*18S rRNA*	1.278	*18S rRNA*	1.177	*18S rRNA*	-	*18S rRNA*	12.000

group. If using a single reference gene, *ALAS1* is considered to be the most stable candidate gene in this group, the second one is *HMBS*. In the CNE group, *18S rRNA* is considered to be the most stable candidate gene, the second one is *ALAS1*. In the FaDu group, *GAPDH* is considered to be the most stable candidate gene, the second one is *HMBS* (**Table 2**).

BestKeeper: Due to the characteristics of the BestKeeper program, only 10 candidate genes can be evaluated at a time. Even though the

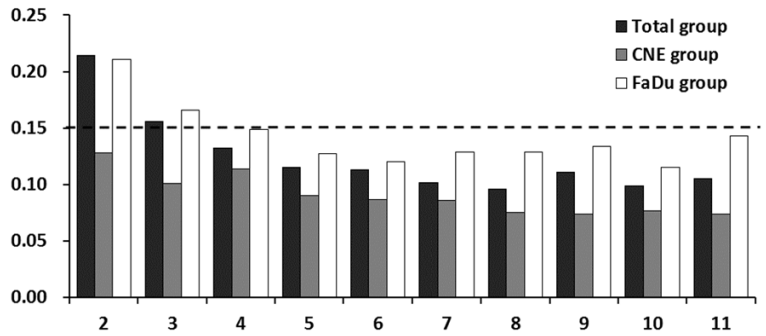

Figure 2. The optimal number of reference genes for normalization. X-axis represents the number of genes that should be used as a combination to achieve a satisfactory accuracy in the relative quantification analysis; Y-axis stands for the pairwise variation value.

top two unstable candidate reference genes ranked by geNorm and NormFinder in the CNE group and FaDu group are the same, in the total group they are not. In order to avoid the standard confusion, we only considered the result of geNorm and removed the top two unstable candidate genes from each group before analyzing. From the results of BestKeeper, we can see that the most stable candidate gene in total group is *ACTB*, the second one is *GAPDH*. In the CNE group, the most stably expressed candidate gene is *HPRT1*, the second one is *GAPDG*. In the FaDu group, the most stably expressed candidate gene is *GAPDH*, the second one is *HPRT1* (**Table 2**).

Final ranking of the candidate reference genes

The M-values obtained from geNorm, stability values obtained from NormFinder, R values obtained from BestKeeper, and their rankings of candidate reference genes obtained from all 3 programs are listed in **Table 2**. Because the rankings of the candidate genes varied slightly, the geometric means of the ranking numbers from 3 programs were calculated to provide an overall ranking of the best candidate reference genes. The smaller geometric mean the candidate gene had the more stably the candidate gene was expressed [26]. The final ranking of the candidate reference genes is also given in **Table 2**. According to the final ranking, *ALAS1* and *HMBS* are considered as the optimal reference genes that can be used in the studies of CNE and FaDu cell lines treated by chemotherapy drugs. *ALAS1* is considered as the optimal reference gene that can be used in the study of CNE with chemotherapy drug treatment, follow by *RPL29*. *GAPDH* is considered as the optimal reference gene that can be used in the study of FaDu with chemotherapy drug treatment, follow by *ALAS1*.

Discussion

In our present study, a systematic evaluation of stability and applicability of reference genes was conducted in order to choose optimal reference genes for performing a more accurate relative quantification analysis of target gene expression in the HNSCC cell line, treated by 5 kinds of chemotherapy drugs. We found that *ALAS1*, *ALAS1* and *GAPDH* were the optimal reference genes for the study of total group, the CNE group, and the FaDu group, respectively. If 2 or more reference genes are needed to achieve better standardization effect, *ALAS1* + *HMBS* + *HPRT1* is considered to be the optimal combination for the total group; *ALAS1* + *RPL29* is considered to be the optimal for the CNE cell line; *GAPDH* + *ALAS1* + *HPRT1* is considered to be the optimal for the FaDu cell line.

The twelve candidate genes in the present study, including *GAPDH*, *ACTB*, *B2M*, *18S rRNA*, *ALAS1*, *RPL29*, *TBP*, *HPRT1*, *PUM1*, *HMBS*, *PPIA*, and *GUSB*, have been commonly used for relative quantification analysis on the study of human tissues or cell lines, and the primer sequences were derived from previous studies [7]. The expression levels of 12 genes determined by qRT-PCR were presented as Cp values. In this study, Cp values of all the samples ranged between 8.25 (*18S rRNA*) and 30.70 (*PUM1*), which is within an acceptable range and could be used as candidate reference genes, as shown in previous studies [27, 28].

To assess more accurate reference gene expression patterns, three specialized programs (geNorm, NormFinder, and BestKeeper) were employed for data analyzing. The results provided are slightly different in the ranking of the candidate gene expression stabilities by the three programs, which is possibly caused by different calculation algorithms [29, 30]. For instance, in total group, geNorm ranked the top four genes, in order, as *HMBS*, *HPRT1*, *ALAS1*,

and *GAPDH*. NormFinder ranked in a different order, as *ALAS1*, HMBS, *GAPDH*, and *HPRT1*. BestKeeper even had an alternative gene, as *ACTB*, *GAPDH*, *ALAS1*, and *HMBS*. In the CNE group, *ALAS1* was ranked first and second by geNorm and NormFinder respectively, but ranked seventh by BestKeeper. However, all three programs produced the same unstable genes. *GUSB* was ranked within the last five in all groups, and *PUM1* was ranked within the last two in all groups. In recent studies, RPL family genes showed a high stability in both human breast cancer cell lines and FFPE biopsies, but *GAPDH* performed poorly [31]. *GAPDH* was also found to be unsuitable in the study of bladder cancer cell lines, in which *B2M* was considered as the most reliable reference gene [32]. In a study of endometrial carcinoma, *PUM1* and *PPIA* showed a high stability, but RPL family genes performed poorly [33]. Taken together, these findings reinforce the notion that the expression of reference genes is context-dependent and has a large variation in different cell types or between treatments in the same cell types. These wide variations may be due to the effect of the toxic effects of chemotherapy drugs on the modulation of gene expressions of the 12 reference genes that have diverse cellular functions. Thus, validating and evaluating the expression stabilities of reference genes and selecting the most stable reference gene is the key to the accuracy of the experiments, before further quantitative studies in different samples or experimental conditions.

Since the expression stabilities of candidate genes were ranked slightly different, the geometric means of the ranking numbers from 3 programs were calculated to provide an overall ranking of the best candidate reference genes. The smaller geometric mean the candidate gene has the more stably the candidate gene was expressed [26]. The final ranking of the candidate reference genes shows that *ALAS1, ALAS1,* and *GAPDH* are suggested to be the optimal reference genes for the study of both CNE and FaDu cell lines together, CNE cell line, and FaDu cell line, respectively. In addition, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines state that normalization can be further improved by using multiple reference genes [34]. If using multiple reference genes to improve accuracy of relative quantification analysis, considering the V value results which is provided by geNorm program, 2-4 reference genes combined together is suggested as optimal for each group. However, according to the standardized principle of relative quantification analysis, this result only provides a guide to decide how many reference genes should be used as a combination for further improving normalization [23], instead of a stringent standard consideration. Previous studies have recommend that a combination of 3 internal control genes is accurate enough for performing a relative quantitative investigation [35]. Comprehensively considering the results of all the 3 programs, it is proposed that the recommended combination for the total group is *ALAS1* + *HMBS* + *HPRT1*; the recommended reference gene combination for the CNE cell line is *ALAS1* + *RPL29*; the recommended reference gene combination for the FaDu cell line is *GAPDH* + *ALAS1* + *HPRT1*.

Conclusions

Twelve candidate reference genes were evaluated under treatment of 5 kinds of chemotherapy drugs in two kinds of HNSCC cell lines. Our present study was committed to analyzing and evaluating the expression stability of reference genes in the chemotherapy drugs that treated HNSCC cell lines. For the simultaneous study of the effects on CNE and FaDu cell lines treated by 5 kinds of chemotherapeutic drugs, *ALAS1* gene or the combination of *ALAS1*, *HMBS* and *HPRT1* gene is considered the most suitable reference gene. For the study of the effects on CNE cell line treated by 5 kinds of chemotherapeutic drugs, *ALAS1* gene or the combination of *ALAS1* and *RPL29* gene is considered the most suitable reference gene. For the study of the effects on FaDu cell line treated by 5 kinds of chemotherapeutic drugs, *GAPDH* gene or the combination of *GAPDH*, *ALAS1* and *HPRT1* gene is considered the most suitable reference gene. This information is important for other researchers that will evaluate mRNA expression on these cell lines and in this experimental condition. It is recommended to select the most stable reference gene before further quantitative studies in diverse samples or experimental conditions. Our recommended reference genes might improve the accuracy in quantitating target gene expression during investigation on

the pharmacological effects and underlying molecular mechanism of chemotherapy drugs on HNSCC.

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Disclosure of conflict of interest

None.

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